Phylogeny of *Rhaponticum* (Asteraceae, Cardueae-Centaureinae) and related genera inferred from nuclear and chloroplast DNA sequence data: taxonomic and biogeographic implications.

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● Background and Aims. The precise generic delimitation of the *Rhaponticum* group is not totally resolved. The lack of knowledge of the relationships between the basal genera of Centaureinae could imply that genera whose position is as yet unresolved could belong to the *Rhaponticum* group. On the other hand, the affinities among the genera that are considered as members of this group are not well-known. The aim of our study is to contribute to the phylogenetic and generic delineation of the *Rhaponticum* group on the basis of molecular data.

● Methods. Parsimony and Bayesian analyses of the combined sequences of one plastid (*trn*L-*trn*F) and two nuclear (ITS region and ETS) molecular markers were carried out. The results of these analyses are discussed in the light of the biogeographic history.

● Key Results. The *Rhaponticum* group appears as monophyletic, and closely related to the genus *Klasea*. Our results confirm the preliminary generic delimitation of the *Rhaponticum* group, with the new incorporation of the genus *Centaurothamnus*. *Ochrocephala* is supported as a separate genus from *Rhaponticum* and, contrary to this, *Acroptilon* and *Leuzea* appear as merged into the genus *Rhaponticum*.

● Conclusions. The new molecular evidence is consistent with the morphological and karyological data, and suggests particularly coherent biogeographic routes of migration and speciation processes for the genus *Rhaponticum*.

Keywords: *Acroptilon*, *Callicephalus*, *Centaurothamnus*, Compositae, ETS, ITS, molecular phylogeny, *Myopordon*, *Ochrocephala*, *Oligochaeta*, *Rhaponticum*, *trn*L-*trn*F

INTRODUCTION

One of the main problems which persists in the subtribe Centaureinae (Asteraceae, Cardueae) is that the phylogenetic relationships between the basal genera are unresolved. Monographers (Dittrich, 1977; Wagenitz and Hellwig, 1996; Garcia-Jacas *et al.*, 2001; Hellwig, 2004; Susanna and Garcia-Jacas, in press) have described informal groups of genera. One of these is the *Rhaponticum* group which comprises about 7 genera and approximately 40 species. Apart from the genus *Rhaponticum* Vaill. (~25 sp.), it includes *Acroptilon* Cass. (2 sp.), *Callicephalus* C. A. Mey. (1 sp.), *Leuzea* DC. (1 sp.), *Myopordon* Boiss. (5 sp.), *Ochrocephala* Dittrich (1 sp.) and *Oligochaeta* (DC.) K. Koch. (4 sp.).

The classic morphological approach limited itself to associate *Rhaponticum* and *Acroptilon* (Dittrich, 1977), and pointed out generic delimitation problems: Holub (1973) concluded that *Leuzea* and *Rhaponticum* should be merged, and Dittrich (1983) that *Rhaponticum imatongensis* (Phillipson) Soják should be segregated to constitute a new monotypic genus: *Ochrocephala*. Later, the molecular approach allowed the addition of *Callicephalus*, *Oligochaeta* (Garcia-Jacas *et al.*, 2001) and *Myopordon* (Susanna *et al.*, submitted).

These genera show the symplesiomorphic characters common to the basal genera of the Centaureinae (homogamous capitula, lack of crystals in the phyllaries, basal hilum, no bolster cells, absence of hairs on the achene and pollen of the *Serratula* type), and some morphological traits which characterize the group:

- a peculiar type of involucral bract with a big, soft scarious, entire or lacerate and usually silvery-white appendage (an exclusive character of the group which, unfortunately, not all the species show);
- dimorphic achenes (the outer dorsiventrally compressed and the inner laterally compressed);
- and finally the double pappus typical of all the Centaureinae, but with the peculiarity that the inner bristles are wider and longer than the outer.

The geographical distributions, environmental conditions and life cycles are very diverse in *Rhaponticum* and related genera. They are naturally distributed in North Africa (including Canary Islands), temperate Eurasia, Siberia and the Far East, Caucasus, Central and Eastern Asia, and Eastern Australia. They grow in deserts or mountains, and are either widely distributed or narrow endemics. They can be perennial or annual, and their habit is shrubby, or hemicryptophyte from 10 cm to more than 1 m in height, or acaulescent. Several species are endangered to the verge of extinction, but one taxon, *Acroptilon*, is considered to be an invasive weed in America and Australia. The *Rhaponticum* group includes the only species of Centaureinae indigenous to Australia: *Rhaponticum australe* (Gaudich.) Soskov (Wagenitz and Hellwig, 1996). Some representatives of the group show medicinal properties which were already known in Roman culture (Plinius, 77), and various species are being marketed due to their anabolic and adaptogenic properties.

The main goals of our study in establishing a combined molecular phylogeny were to: (1) elucidate the relationships between the basal groups of Centaureinae, with the purpose of determining the taxa most closely related to the *Rhaponticum* group; (2)

verify the generic delimitation of the *Rhaponticum* group and the relationships between its genera; (3) link our findings to the group's biogeographic history.

MATERIAL AND METHODS

Plant Material

The sampling includes representatives of all the genera of the *Rhaponticum* group, and all the species of the genus *Rhaponticum* (except *R. namanganicum* Iljin). The outgroups have been selected according to previous works by Garcia-Jacas *et al.* (2001) and Susanna *et al.* (submitted) to represent most of the basal Centaureinae which could have phylogenetic affinities with our focus of study: *Centaurothamnus* Wagenitz & Dittrich, *Cheirolophus* Cass., *Klasea* Cass., *Plagiobasis* Schrenk, *Psephellus* Cass., *Rhaponticoides* Vaill., *Serratula* L. and *Stizolophus* Cass. The purpose in representing numerous outgroups is to permit us, without forcing the topology, to test the good assignation of a taxon as outgroup or as ingroup, and to define the more closely related taxa to the *Rhaponticum* group. Both previously published and the 111 new sequences (31 ITS, 47 ETS, 33 *trn*L-*trn*F) were used in the analyses. The origin of the samples and GenBank sequence accession numbers are given in Table 1.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted following the miniprep procedure of Doyle and Doyle (1987) as modified by Soltis *et al.* (1991) and Cullings (1992) from herbarium material, silica-gel-dried leaves collected in the field, or fresh leaves of plants cultivated

in the Botanic Institute of Barcelona. DNA of old herbarium material was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA).

nrDNA ITS region strategies. ITS1, 5.8S gene and ITS2 (the ITS region) were amplified and sequenced together with 1406F (Nickrent *et al.*, 1994), ITS1 (White *et al.*, 1990) and 17SE (Sun *et al.*, 1994) as forward primers, and ITS4 (White *et al.*, 1990), 26SE (Sun *et al.*, 1994) as reverse primers, referring to the protocol described in Soltis and Kuzoff (1993). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Direct sequencing of the amplified DNA segments was performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA). Nucleotide sequencing was carried out at the Serveis Cientificotècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, CA).

nrDNA ETS region strategies. The ETS region was amplified and sequenced with ETS1f as forward primer and 18S-2L as reverse primer (Linder *et al.*, 2000), referring to the PCR procedure described in the same publication. Purification and direct sequencing of the amplified DNA segments were performed as for the ITS region.

cpDNA trn*L*-trn*F region strategies.* The *trn*L-*trn*F region includes the *trn*L intron, the 3' *trn*L (UAA) exon, and the intergenic spacer between *trn*L (UAA) and *trn*F (GAA), which were amplified and sequenced together. The universal primers *trn*L-c, forward, and *trn*L-f, reverse (Taberlet *et al.*, 1991) were used to amplify and sequence the *trn*L-F region. For old material we have amplified and sequenced the region in two parts using the two precedent primers and the *trn*L-e, forward, and *trn*L-d, reverse, of the same author. The PCR procedure includes a warm start at 95°C for 1 min 35 s, followed by 80°C during which the polymerase (Ecotaq, Ecogen S.R.L., Barcelona, Spain) is added,

and 34 cycles of 1 min denaturation at 93°C, 1 min annealing at 58°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. Purification and direct sequencing of the amplified DNA segments was performed as for the ITS region.

Phylogenetic Analyses

Nucleotide sequences were edited with Chromas 1.56 (Technelysium Pty., Tewantin, Australia). DNA sequences were aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990).

Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b4a (Swofford, 1999) using Tree Bisection Recognition (TBR) branch swapping with character states specified as unordered and unweighted. All most-parsimonious trees (MPT) were saved. To locate islands of most-parsimonious trees (Maddison, 1991), we performed 100 replicates with random taxon addition, also with TBR branch swapping. Trees lengths, consistency index (CI) and retention index (RI) are always given excluding uninformative characters. Bootstrap (BS; Felsenstein, 1985) was carried out to obtain support estimates of the nodes of the trees selected. Bootstrap analysis was performed using 1000 replicates of heuristic search with the default options. ACCTRAN (accelerated transformation) character-state optimization was used for all illustrated trees. In order to conserve the phylogenetic information of insertions-deletions and at the same time avoiding over-estimation of lengthy indels, we have used "missing data" and coded the indels as presence-absence characters added to the end of the matrix.

Bayesian analysis: data sets were analysed using Modeltest 3.04 (Posada and Crandall, 1998) to determine the sequence evolution model that best described our data. This

model was used to perform a Bayesian analysis using the program Mr. Bayes 3.0 (http://morphbank.ebc.uu.se/mrbayes/; Huelsenbeck *et. al.*, 2001). Four Markov chains were run simultaneously for 1.000.000 generations, and these were sampled every 100 generations. Data from the first 1000 generations were discarded as the "burn-in" period, after confirming that likelihood values had stabilized prior to the 1000^{th} generation. The 50% majority rules consensus phylogeny and posterior probability of nodes (PP) were calculated from the remaining sample.

Biogeographic distributions: the distributions were mapped on the tree using the Farris double pass method (Farris, 1970) which provides the hypothesized distributions of the deep branches and nodes. Each taxon branch and internode was colored as to its distribution using Adobe illustrator.

RESULTS

Some of our ETS sequences have repeats in the 5' end of the region, as found in other groups (Baldwin and Marcos, 1998; Linder *et al.*, 2000). In most cases, these repetitions constitute autapomorphic events or characterize a group without alignment or homology problems. Conversely, the majority of *Klasea* species present a region with a different number of repetitions. Because of these repetitions, we have not obtained the entire sequence for some species; and for the others their alignment was impeded by the difficulty of establishing the homology of the repetitions. For these reasons, this repeated zone of *Klasea* was removed from the matrix.

Parsimony analysis: the results from the combined ITS, ETS and *trn*L-*trn*F parsimony analysis are given in Table 2.

Bayesian analysis: the GTR+G+I model was found to be the most efficient model for optimizing sequence evolution of our data set.

The two methods of phylogeny reconstruction lead to congruent results (there is no discordance for strong supported branches) that lead us to present only the tree obtained with the Bayesian inference, shown in Fig. 1. This tree indicates both the bootstrap values (calculated by the parsimony analysis), the posterior probability (calculated by the Bayesian inference), and the biogeography mapping.

DISCUSSION

This new molecular phylogeny allows us to draw some hypotheses regarding the evolutive history of the *Rhaponticum* group. Among all the genera selected to constitute the outgroup, the genus *Klasea* appears as the most closely related to the *Rhaponticum* group (PP=97 %, BS=81 %, Fig. 1). Furthermore, our results contradict the election of *Centaurothamnus* as outgroup, and suggest that it should be included in the *Rhaponticum* group. On the other hand, all the taxa previously considered as members of the *Rhaponticum* group appear in the ingroup (PP=98 %, Fig. 1).

Klasea, sister of *Rhaponticum* group ?

Klasea (≡ *Serratula* section *Klasea*) comprises ca. 65 species distributed from the Western Mediterranean to China and the Western part of the Himalayas. The phylogenetic distance within the genus *Serratula* between the *Serratula* sensus stricto (*S. coronata* L. and *S. tinctoria*) and the *Klasea* section, has been demonstrated by different authors, on morphological (Wagenitz and Hellwig, 1996) and on molecular

bases (Martins and Helllwig, 2003). Therein, those authors were in accordance with Cassini (1825) who placed *Klasea* as a distinct genus from *Serratula*. Our results confirm these findings and, furthermore, clearly define *Klasea* as closely related to the *Rhaponticum* group (PP=97 %, BS=81 %, Fig. 1). This proximity was previously suggested by Lessing (1832), who considered *Klasea* Cass. as a subgenus of *Rhaponticum*. Moreover, the observation of the achene morphology has brought to light for the first time the fact that *Klasea* shows the same achene polymorphism as the *Rhaponticum* group. The *Klasea* species constitute a monophyletic group (PP=99 %, BS=100 %, Fig. 1). Among them, *Serratula algida* Iljin, *S. chartacea* C. Winkl., *S. biebersteiniana* (Iljin ex Grossh.) Takht. and *S. kuzhistanica* (Mozaffarian) Mozaffarian have not been recombined as *Klasea* until now; this has lead us to establish new nomenclatural combinations detailed in the appendix.

The monophyly of the *Rhaponticum* group is supported by the Bayesian inference (PP=98 %, Fig. 1), but the parsimony analysis shows an irresolution between *Klasea*, the *Centaurothamnus* plus *Ochrocephala* clade, and the rest of the genera. Even though the parsimony advices us to hesitate before placing *Klasea* as sister to the *Rhaponticum* group, this hypothesis is, nonetheless, supported both by karyological and morphological data. Representatives of the genus *Klasea* have a base chromosome number of x=15 (Löve and Löve, 1961; Cantó, 1982 and 1984; Garcia-Jacas *et al.*, 1998a and 1998b), while *Centaurothamnus* and *Callicephalus* have x=14 (Wagenitz *et al.*, 1982 for the former; Chouksanova *et al.*, 1968; Hellwig, 1994; Garcia-Jacas *et al.*, 1998a, for the second one), and *Acroptilon*, *Leuzea* and most of the *Rhaponticum* species present x=13 (Hidalgo, unpubl. res.). Descending dysploidy being one of the

main evolutionary mechanisms in plants, and in particular in the Centaureinae (Fernández Casas and Susanna, 1986; Garcia-Jacas *et al.*, 1996; Vilatersana *et al.*, 2000), this could suggest that x=15 is more primitive than x=14. Then, in the case of our focus of study, this could suggest that *Klasea* should be sister to the *Rhaponticum* group. Furthermore, although *Klasea* shows achene polymorphism as the *Rhaponticum* group, it does not exhibit the two other morphological apomorphies which define it, namely the typical involucral bracts (present in *Ochrocephala*) and the characteristic pappus (present in *Centaurothamnus* and *Ochrocephala*).

Centaurothamnus and *Ochrocephala*:

Our results confirm that *Ochrocephala* is more appropriately treated as a monotypic genus (Dittrich 1983), rather than considered as *Rhaponticum imatongensis*. Furthermore, this study allows us to define for the first time the systematic position of the genus *Centaurothamnus*. This genus was placed with the genera of «uncertain position» by Wagenitz & Hellwig (1996), Garcia-Jacas *et al.* (2001) and Hellwig (2004). With the new molecular evidence, *Centaurothamnus* appears as closely related to the genus *Ochrocephala* (PP=99 %, BS=100 %, Fig. 1). This result is unexpected but not surprising, because these two monotypic genera are geographically very close: *Centaurothamnus maximus* Wagenitz & Dittrich grows in Southwestern Arabia, in Yemen, and *Ochrocephala imatongensis* (Phillipson) Dittrich in Eastern Africa (Ethiopia, Sudan and Congo). Morphologically, these two taxa share the same shrubby habit, an exclusive trait of the group. The shrubby port is uncommon within the Centaureinae, and outside *Centaurothamnus* and *Ochrocephala* it is only know from *Centaurodendron* Johow, *Centaurea ptosimopappa* Hayek and *Cheirolophus*. It

corresponds, probably, to a secondary adaptation, this phenomenon being particularly evident for the insular taxa such as *Centaurodendron* and *Cheirolophus* (Böhle *et al.*, 1996; Garnatje *et al.* unpubl. res.). In the case of *Centaurothamnus* and *Ochrocephala* it could be a secondary adaptation of hemicryptophytes colonizing extremely dry habitats, where they suffer from physiologic constraints and lack of pollinators. *Centaurothamnus* and *Ochrocephala* are genetically and morphologically distinguished: their molecular divergence for the three markers considered is 13.8 %; *Ochrocephala* shows the typical involucral bract appendages of the *Rhaponticum* group, while these are not present in *Centaurothamnus*. A new question induced by these results could be to determine whether *Centaurothamnus* and *Ochrocephala* should be more appropriately maintained as distinct genera or whether they should be merged to constitute a single genus.

Callicephalus nitens:

The genus *Callicephalus* includes a single species, *Callicephalus nitens* (M. Bieb. ex Willd.) C. A. Mey., from the middle and low mountains of the Caucasus, Central Asia, and the Near East. It appeared within the *Rhaponticum* group in the molecular analysis of Garcia-Jacas *et al.* (2001), but with a weak statistical support. Our results strongly support the fact that *Callicephalus* belongs to the *Rhaponticum* related genera (PP=99 %, BS=86 %, Fig. 1). Because of its annual nature, this species shows a high rate of mutation, which could have induced distortions in the parsimony analysis. However, the Bayesian inference method, less affected by the long branch attraction, leads to an identical result. Furthermore, this hypothesis is reinforced by morphological features such as the structure of the inner pappus or the tuberculate pericarp (Garcia-Jacas *et al.*

2001). *Callicephalus* has no closely related taxa and appears as isolated in the phylogeny. Thus, this genus should be one of the numerous «relict» taxa which grow in the Caucasus. The abundance of relict and endemic plant species in this area seems largely due to the fact that it was spared glaciation during the most recent Iceages.

The rest of the ingroup belongs to a strongly supported clade (PP=99 %, BS=100 %, Fig. 1) which includes the genera *Acroptilon*, *Leuzea*, *Myopordon*, *Oligochaeta*, and *Rhaponticum*, placed in three different groups. The relationships between these three groups are not resolved either in the parsimony or the Bayesian inference.

Oligochaeta and *Myopordon:*

Myopordon, a small genus with 5 perennial species from the Near East which had been placed in the Carduinae, and *Oligochaeta*, another genus composed of 4 annual species from the Near East, Caucasus, Afghanistan and India, and related to *Rhaponticum*, have apparently nothing in common. However their close relationship was put in evidence in the molecular study of Susanna *et al.* (submitted), and is also confirmed in this analysis (PP=99 %, BS=96 %, Fig. 1), whose sampling of the *Rhaponticum* group species is much more complete. In spite of the morphological review of *Myopordon* by Wagenitz (1958), several questions remain open. One consists of the generic delimitation of *Myopordon* and *Oligochaeta*: our analyses support the monophyly of *Oligochaeta* (PP=100 %, BS=100 %, Fig.1) but not that of *Myopordon*. More studies are necessary to verify whether *Myopordon* and *Oligochaeta* are independent taxa or whether they should be merged. Another question could be the morphological traits of this clade, focusing especially on their palynological characteristics, which are baffling. While all

the species of the *Rhaponticum* group show a *Serratula*-type pollen, *Oligochaeta* presents a reduced form of *Serratula*-type pollen grain (Villodre and Garcia-Jacas, 2000), and *Myopordon* exhibits 3 different pollen types: *Jacea*, *Centaurea scabiosa* and *Serratula* (Wagenitz, 1958). An additional question would be to understand how these two specialized and divergent taxa can be so narrowly related to *Rhaponticum* up to the point that our three molecular markers are not able to segregate them.

The genus *Rhaponticum*:

The genus *Rhaponticum* does not appear as monophyletic in the phylogeny established for the following two reasons. Firstly, its segregation from the clade of *Myopordon* plus *Oligochaeta* is not statistically supported (Fig. 1). Fortunately, the resolution within the genus *Rhaponticum* is better, and shows two strongly supported clades, one oriental (PP=99 %, BS=100 %, Fig. 1) and the other occidental (PP=99 %, BS=92 %, Fig. 1). The second reason lead us to the paraphyly of *Rhaponticum* in its present circumscription: this is, that the genera *Acroptilon* and *Leuzea* are firmly nested in the genus *Rhaponticum*, the first in the oriental clade, and the second in the occidental clade (Fig. 1). This implies some nomenclatural changes to reconcile the delimitation of the genus with this new evidence. Another evidence for the placement of *Acroptilon* and *Leuzea* in the genus *Rhaponticum* is that the three taxa share the same chromosome number x=13 (Hidalgo, unpubl. res.), which is uncommon within the Centaureinae.

The comparison between the more compehensive infrageneric classification of *Rhaponticum* (Holub, 1973) and the molecular phylogeny shows numerous incongruences (Fig. 1). Only two of the seven subgenera described are natural groups: the subgenus *Rhaponticella* (Soskov) Holub (PP=99 %, Fig. 1) and the subgenus *Leuzea*

DC. (PP=99 %, BS=99 %, Fig. 1). Our results suggest that the genus *Rhaponticum* should be more appropriately divided into only two subgenera, these corresponding to the oriental and the occidental clades. Nevertheless, we have been unable to detect any character for defining either group on morphological grounds.

The *Rhaponticum* oriental clade:

The first clade (PP=99 %, BS=100 %, Fig. 1) consists mostly of Central Asian species, but includes species from Middle and Eastern Asia, Australia and the Eastern Europe. These species have relatively restricted areas of distribution, except two groups of taxa which have wider areas.

(1) One group extends from the Western to Eastern Europe, and is constituted by *Rhaponticum serratuloides* (Georgi) Bobrov and *Acroptilon* (PP=99 %, BS=100 %, Fig. 1). The incorporation of *Acroptilon* in *Rhaponticum* had never been mentioned before, and this implies new nomenclatural combinations detailed in the appendix. The most recent classifications do not recognize *Acroptilon australe* Iljin as a separate species from *A. repens* (L.) DC (Hellwig, 2004; Susanna and Garcia-Jacas, in press), while on the contrary Soskov (2001) considers them as two well-defined species. Our results do not allow us to pronounce a verdict, and more studies are necessary to clarify the status of *A. repens*. This is the reason why we have preferred to abstain from making a new combination for this taxon. *Acroptilon* is considered as an invasive weed in America and Australia, where it adversely affects agronomic harvests. It is agressively competitive and exhibits allelopatic effects. It differs from the other species of the group, most of them endemics restricted to unfavorable environments where the competition with other species is less notable, as for example the mountain screes. The

structure of capitula, achenes and the type of ramification are basically the same as *Rhaponticum*, but *Acroptilon* shows secondary adaptations due to its colonizing strategy: it is a hemicryptophyte like *Rhaponticum* but, instead of presenting few stems weakly or not ramified, this species generates numerous strongly-branched stems in spring, due to its extensive root and rhizome system. Therefore, vegetative multiplication is favoured, although it also produces numerous capitula and achenes.

(2) The other group expands from Central to Eastern Asia and Australia and is composed of *Rhaponticum australe* and *R. uniflorum* (L.) DC. The close relationship between these two species (PP=100 %, BS=97 %, Fig. 1) is a logical result, considering that *R. uniflorum* is the only species of the genus which has reached Eastern Asia. From a geographic point of view, this was the better candidate as sister to the Australian species. The fact that *R. australe* is the only species of Centaureinae indigenous to Australia is surprising because nothing explains such a long dissemination distance of the achenes of a *Rhaponticum* species. This lead Susanna and Garcia-Jacas (in press) to hypothesize that the species was doubtfully native in Australia. There is a considerable genetic divergence between *R. uniflorum* and *R. australe* for the three studied regions (8.7 %), and this allows us to rule out the possibility of a recent introduction from *R. uniflorum*. The colonizing event would have taken place during the period of lowermost sea level (between 50,000 and 84,000 years ago), from the coasts of South Asia. Was it the Aborigines that introduced the plant, and were they motivated by its medicinal properties ? Had the species, on the other hand, reached Australia without human intervention, we would need to ask ourselves how ?

The *Rhaponticum* occidental clade:

This second clade within *Rhaponticum* (PP=99 %, BS=92 %, Fig. 1) embraces species distributed in North Africa, Canary Islands, Europe and the Near East. *Rhaponticum pulchrum* Fisch. & C. A. Meyer, from Iran-Afganistan and the Caucasus, is situated as sister of the remainder of this group (Fig. 1), which suggests that it originated in the Near East. One characteristic of the occidental clade is that we do not see a grouping of the North-African species in one subclade, and the European species in another subclade, but, on the contrary, various subclades combining species from North Africa, Europe, and/or a mixed distribution (Fig. 1). This suggests several independent passages from one continent to the other during the evolutionary history of the group:

(1) the association *Rhaponticum heleniifolium* Godr. & Gren. plus *R. cynaroides* Less. (PP=99 %, BS=100 %, Fig. 1) is the exception because the former one is endemic of the Alps and the latter of the Pyrenees. The two species present the particularity of exhibiting ramified inflorescential stems. Few species of *Rhaponticum* show this character, and those are always less than 50 cm in height, while *R. heleniifolium* and *R. cynaroides* reach 1 m.

(2) one of these passages could be the case for the group including the *Leuzea* species (PP=99 %, BS=99 %, Fig. 1). Our study confirms, for the first time on a molecular basis, that *Leuzea* and *Rhaponticum* should be fused, as previously suggested by Holub (1973) on morphological bases. *Leuzea berardioides* Batt., endemic to the High Atlas (Morocco), appears as clearly segregated from *L. conifera* (L.) DC. (the molecular divergence for the studied regions between the two species is 7 %). This fact contradicts its consideration as a synonym or as a subspecies of *L. conifera* by Susanna (2002), and Greuter (2003), respectively, and implies a new nomenclatural combination of *L. berardioides* as *Rhaponticum*, as detailed in the appendix. Another taxon, *L. fontqueri*,

had been described by Sauvage (1968) as closely related to *L. berardioides*. Our results suggest that *L. fontqueri* is more closely related to *L. conifera* (PP=100 %, BS=100 %, Fig. 1), the only molecular divergences observed between these two taxa concerning polymorphic positions. *Leuzea conifera* presents a wide distribution area (W Mediterranean and Portugal) and a high morphological variability. In this sense, more studies are necessary to determine whether the observed differences with *L. fontqueri* are included in the natural variability of *L. conifera*, or if this endemic of the Chefchaouène Mountains (Morroco) merits the status of species. Meanwhile, the new nomenclatural combination for *L. fontqueri* as *Rhaponticum* is proposed in the appendix. *Leuzea conifera* had been previously combined as *Rhaponticum* by Greuter (2003). *Rhaponticum acaule* (L.) DC., is positioned at the base of the *Leuzea* group, but without statistical support.

(3) *Rhaponticum canariense* DC., the only representative of *Rhaponticum* from the Canary Islands and seriously threatened with extinction, appears closely related to *R. exaltatum* (Willk.) Greuter, a species from the Central Spain and North-East Portugal (PP=99 %, BS=100 %, Fig. 1). Although floras usually do not indicate it, *R. exaltatum* could also be present in Morocco, in the Rif Atlas, according to a voucher from the Herbarium of Montpellier ["montagnes de Ketama", Sennen & Mauricio, VI-1934 (MPU) ; the determination of the herbarium sample was established and confirmed respectively by Maire in 1936 and Dittrich in 1976].

(4) There is another subclade which associates *R. scariosum* Lam. and *R. longifolium* (Hoffmanns. & Link) Dittrich (PP=99 %, BS=100 %, Fig. 1). *Rhaponticum cossonianum* (Ball) Greuter is positioned as sister of these two species, but with a weak statistical suppport (Fig. 1).

An important outcome of this study is that the two representatives of *Rhaponticum* growing in the Alps, *R. scariosum* and *R. heleniifolium*, considered by several authors as subspecies of *R. scariosum* (Briquet 1902; Rouy 1905; Burnat 1931; Holub 1973; Dostál 1976), do not appear as sisters in the phylogeny (Fig. 1). This implies *inter alia* that the colonization of the Alps took place in, at least, two independent events. In the same order of things, the biogeographic inference suggests that the two species of *Rhaponticum* indigenous to the East of Europe (*R. serratuloides* and *R. scariosum*), could have reached this region in two different ways: one expansion across Central Asia to Eastern Europe generating *R. serratuloides*; the other expansion through the Near East, North Africa and then to the Iberian Peninsula, thence on to the Alps, generating *R. scariosum.*

In view of this interesting hypothesis suggested by our analysis, it is regrettable that some nodes of the *Rhaponticum* occidental clade are weakly supported, and it would be interesting to perform more studies towards a better understanding of its biogeographic history.

To conclude, this study fulfills our main expectations. It defines the genus *Klasea* as being probably the most closely related group of taxa to the *Rhaponticum* group. The generic delimitation of the *Rhaponticum* group would include the genera *Callicephalus*, *Centaurothamnus*, *Myopordon*, *Ochrocephala*, *Oligochaeta*, and *Rhaponticum* (including *Acroptilon* and *Leuzea*). The new molecular evidence is consistent with the karyological and morphological data, and suggests particularly coherent biogeographic routes of migration and speciation processes for the genus *Rhaponticum.*

APPENDIX

Klasea Cass., in Cuvier, Dict. Sci. Nat. 35: 173 (1825).

K. algida (Iljin) Hidalgo comb. nov. ≡ *Serratula algida* Iljin, *Repertorium Specierum Novarum Regni Vegetabilis* 35: 928-935 (1934).

K. chartacea (C. Winkl.) Hidalgo comb. nov. ≡ *Serratula chartacea* C. Winkl., in Tr. Peterb. Bot. Sada 9,2: 524 (1886).

K. biebersteiniana (Iljin ex Grossh.) Hidalgo comb. nov. ≡ *Serratula radiata* ssp. *biebersteiniana* Iljin ex Grossh., Fl. Kavk. 4 (1934) ≡ *Serratula biebersteiniana* (Iljin ex Grossh.) Takht., in Takht. et Fed., Fl. Erevana : 323 (1945).

K. kuzhistanica (Mozaffarian) Mozaffarian comb. nov. ≡ *Centaurea khuzistanica* Mozaffarian, *Iranian Journal of Botany* **5**(2): 84 (1992) ≡ *Serratula khuzistanica* (Mozaffarian) Mozaffarian in Garcia-Jacas *et al.*, *Botanical Journal of the Linnean Society* **128**: 420 (1998).

Rhaponticum Adans.

R. berardioides (Battand.) Hidalgo, comb. nov. ≡ *Leuzea berardioides* Batt., Contr. Fl. Alt., 55 (1919) ≡ *Rhaponticum coniferum* (L.) Greuter subsp. *berardioides* (Battand.) Greuter, *Willdenowia* **33**:61 (2003).

R. fontqueri (Sauvage) Hidalgo, comb. nov. ≡ *Leuzea Fontqueri* Sauvage, *Collectanea Botanica* **59**, 7(2) : 1100-1104 (1968).

R. repens (L.) Hidalgo, comb. nov. ≡ *Centaurea repens* L., Species Plantarum ed. 2, 1293 (1763) ≡ *Acroptilon repens* (L.) DC., Prodromus 6: 663 (1838).

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1 TABLE 1. Origin of the materials, herbaria where the vouchers are deposited and

2 GenBank accession numbers.

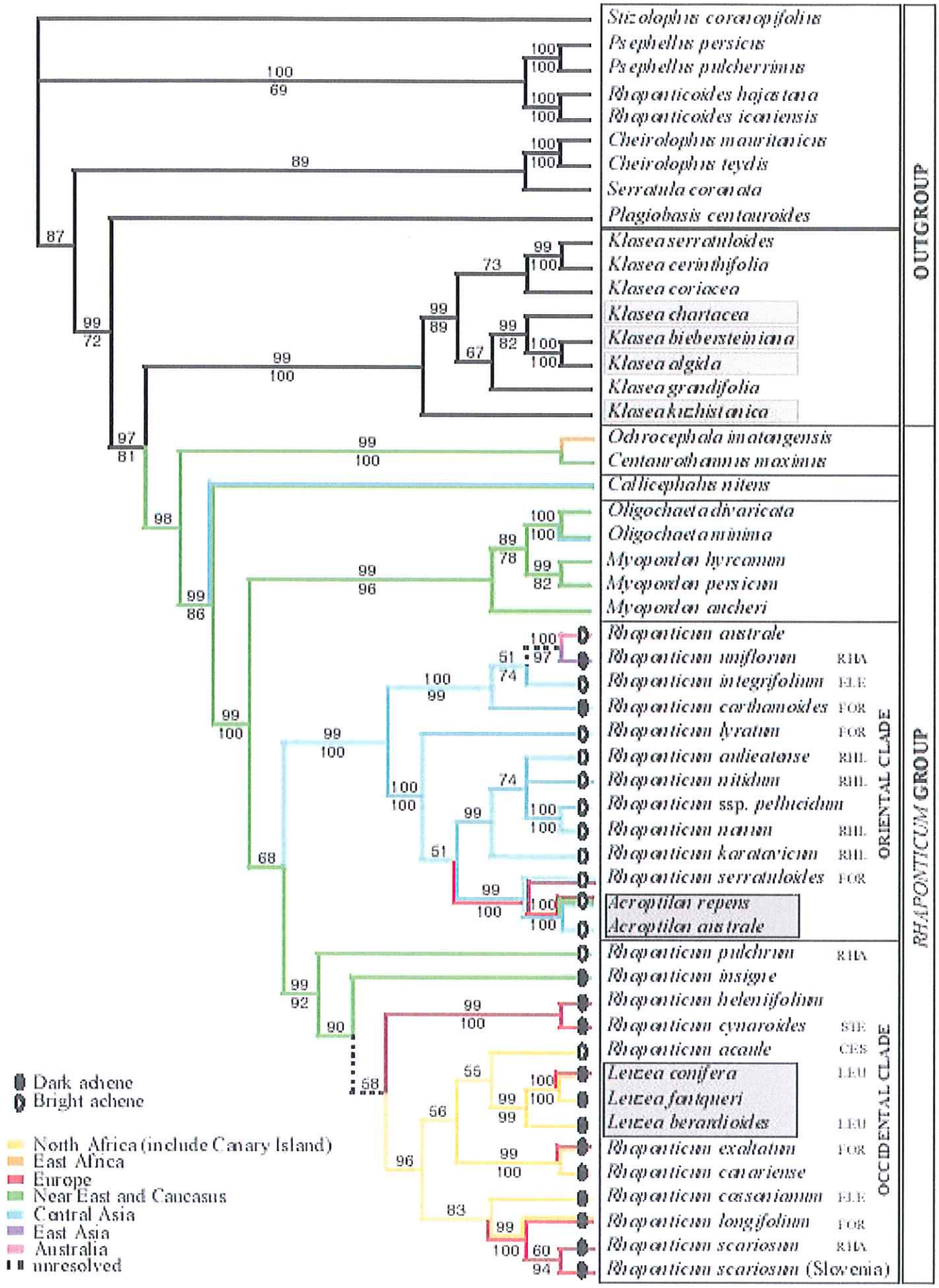
SPECIES	VOUCHER	ITS ACCESSION	ETS ACCESSION	trnL-F ACCESSION
Acroptilon australe Iljin	MONGOLIA: *V. Grubov 301 et al.* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Acroptilon repens (L.) DC.	UZBEKISTAN: *Susanna 2046 et al.* (BC)	AY826223	xxxxxxxx	AY772268
Callicephalus nitens (M. Bieb. ex Willd.) C. A. Mey.	ARMENIA: *Susanna 1578 et al.* (BC)	AY826237	xxxxxxxx	AY772281
Centaurothamnus maximus Wagenitz & Dittrich	YEMEN: *Molero s. n.* (BC)	AY826259	xxxxxxxx	AY772301
Cheirolophus mauritanicus (Font Quer) Susanna	MOROCCO: *Romo 4617 et al.* (BC)	AY826261	xxxxxxxx	AY772303
Cheirolophus teydis (C. Sm.) G. López	SPAIN: *Susanna 1429 et al.* (BC)	AY826262	xxxxxxxx	AY772304
Klasea algida (Iljin) Hidalgo	TAJIKISTAN: *Susanna 2558 & Romaschenko* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea biebersteiniana (Iljin ex Grossh.) Hidalgo	ARMENIA: *Susanna 1493 et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea cerinthifolia (Sm.) Greuter & Wagenitz	IRAN: *Susanna 1700 et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea chartacea (C. Winkl.) Hidalgo	TAJIKISTAN: *Susanna 2467 & Romaschenko* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea coriacea (Fisch. & Mey. ex DC.) Holub	ARMENIA: *Susanna 1558 et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea grandifolia (P. H. Davis) Greuter & Wagenitz	IRAN: *Susanna 1709 et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea kuzhistanica (Mozaffarian) Mozaffarian	IRAN: *Mozaffarian 70181* (TARI)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Klasea serratuloides (DC.) Greuter & Wagenitz	ARMENIA: *Susanna 1569 et al.* (BC)	AY826295	xxxxxxxx	AY772334
Leuzea berardioides Batt.	MOROCCO: *Hidalgo & Romo 12749* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Leuzea conifera (L.) DC.	SPAIN: *Font s. n.* (BC)	AY826298	xxxxxxxx	AY772337
Leuzea Font-Queri Sauvage	MOROCCO: *Hidalgo & Romo 12621* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Myopordon aucheri Boiss.	IRAN: *Carls s.n.* (W)	AY826299	xxxxxxxx	AY772338
Myopordon hyrcanum (Bornm.) Wagenitz	IRAN: *Koelz 16395* (W)	AY826300	xxxxxxxx	AY772339
Myopordon persicum Boiss.	IRAN: *Remandieri s.n.* (W)	AY826301	xxxxxxxx	xxxxxxxx
Ochrocephala imatongensis (Phillipson) Dittrich	ETHIOPIA: *Fantahun Simon 9163 et al.* (K)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Oligochaeta divaricata (Fisch. & C. A. Mey.) K. Koch	ARMENIA: *Susanna 1583 et al.* (BC)	AY826306	xxxxxxxx	AY772344
Oligochaeta minima (Boiss.) Briq.	UZBEKISTAN: *Botanical Garden of Tashrent* (BC)	AY826307	xxxxxxxx	AY772345
Plagiobasis centauroides Schrenk	KAZAKHSTAN: *Susanna 2130 et al.* (BC)	AY826312	xxxxxxxx	xxxxxxxx
Psephellus persicus (DC.) Wagenitz	IRAN: *Susanna 1716 et al.* (BC)	AY826316	xxxxxxxx	AY772352
Psephellus pulcherrimus (Willd.) Wagenitz	ARMENIA: *Susanna 1492 et al.* (BC)	AY826317	xxxxxxxx	AY772353
Rhaponticoides hajastana (Tzvelev) M. V. Agab. & Greuter	ARMENIA: *Susanna 1587 et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticoides iconiensis (Hub.-Mor.) M. V. Agab. & Greuter	TURKEY: *Ertugrul 1761* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum acaule (L.) DC.	ALGERIA: *Montserrat 2331 et al.* (BC)	AY826334	xxxxxxxx	AY772369
Rhaponticum aulieatense Iljin	KYRGYZSTAN: *Sheremetova & Lazkov* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum australe (Gaudich.) Soskov	AUSTRALIA: *Funck 12203* (BC)	AY826335	xxxxxxxx	AY772370
Rhaponticum canariense DC.	SPAIN: *Carqué Alamo s. n.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum carthamoides (Willd.) Iljin	RUSSIA: *Botanical Garden of Sibiricus Centralis, Novosibirsk 2003/2004-1062* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum cossonianum (Ball) Greuter	MOROCCO: *Gómiz* 17-IV-2003 (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum cynaroides Less.	SPAIN: *Hidalgo 504, et al.* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx

Rhaponticum exaltatum (Willk.) Greuter	SPAIN: *Garcia-Jacas & Susanna 2434* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum heleniifolium Godr. & Gren.	*Botanical Garden of Minsk, Bielorussia 1/303-2000* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum insigne (Boiss.) Wagenitz	IRAN: *Archibald 2034* (K)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum integrifolium C. Winkl.	TAJIKISTAN: *Makhmetov & R. Kamelin 344* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhapontikum karatavicum Iljin	KAZAKHSTAN: *Kamelin* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum longifolium (Hoffmanns. & Link) Dittrich	PORTUGAL: *Garcia-Jacas & Susanna 2436* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum lyratum C. Winkl. ex Iljin	TAJIKISTAN: *Kossov* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum nanum Lipsky	TAJIKISTAN: *Kochkariova 5834* (DUSH)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum nanum Lipsky ssp. *pellucidum* (Rech. f.) Dittrich	AFGHANISTAN: *Renz 120* (W)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum nitidum Fisch.	RUSSIA: *Kalibernova 5676, et al.* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum pulchrum Fisch. & C. A. Meyer	RUSSIA: *326 et al.* (LE)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum scariosum Lam.	SLOVENIA: *Botanical Garden of Universitatis Labacensis Slovenia 1994-180* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
	Botanical Garden of Minsk, Bielorussia 1/304-2000 (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum serratuloides (Georgi) Bobrov	*Botanical Garden of Cluj-Napoca, Romania 636-2001* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Rhaponticum uniflorum (L.) DC.	MONGOLIA: *Vallès 13-2003* (BC)	xxxxxxxx	xxxxxxxx	xxxxxxxx
Serratula coronata L.	*Botanical Garden of Vienna, Austria* (BC)	AY826327	xxxxxxxx	AY772362
Stizolophus coronopifolius Cass.	TURKEY: *Ilarslan 4303* (ANK)	AY826337	xxxxxxxx	AY013516

TABLE 2. Results from the combined ITS, ETS and *trn*L-*trn*F parsimony analysis. The consistency and homoplasy indexes are calculated excluding uninformative characters.

Data set	Combined analyses
Total characters	2950 [ITS: 669, ETS: 1354, *trn*L-*trn*F: 927]
Informative substitutions	549 [ITS: 149, ETS: 375, *trn*L-*trn*F: 25]
Number of MTPs	558
Number of steps	1582
Consistency index (CI)	0.48
Retention index (RI)	0.72
Mean pairwise distances, ingroup (%)	from 0 [*Acroptilon australe*/*A. repens*; *Leuzea conifera*/*L. fontqueri*] to 37 [*Acroptilon australe*, *A. repens*/*Callicephalus nitens*]

FIG1. Majority-rule consensus tree based on Bayesian analyse. Numbers above branches indicate Bayesian clade-credibility values (Posterior probability); Numbers below branches indicate parsimony bootstrap percentages. Subgenus of *Rhaponticum*: CES=*Cestrinus*; ELE=*Eleutherochaetum*; FOR=*Fornicium*; LEU=*Leuzea*; RHA=*Rhaponticina*; RHL=*Rhaponticella*; STE=*Stemmacantha*.


Stizolophus coronopifolius
Psephellus persicus
Psephellus pulcherrimus
Rhaponticoides hajastana
Rhaponticoides iconiensis
Cheirolophus mauritanicus
Cheirolophus teydis
Serratula coronata
Plagiobasis centauroides
OUTGROUP
Klasea serratuloides
Klasea cerinthifolia
Klasea coriacea
Klasea chartacea
Klasea biebersteiniana
Klasea algida
Klasea grandifolia
Klasea kuchistanica
Odrocephala imatangensis
Centaurothamnus maximus
Callicephalus nitens
Oligochaeta divaricata
Oligochaeta minima
Myopordon lyrcanum
Myopordon persicum
Myopordon aucheri
Rhaponticum australe
Rhaponticum uniflorum RHA
Rhaponticum integrifolium ELE
Rhaponticum carthamoides FOR
Rhaponticum lyratum FOR
Rhaponticum adlieatense RHL
Rhaponticum nitidum RHL
Rhaponticum ssp. pellucidum
Rhaponticum nanum RHL
Rhaponticum karatavicum RHL
Rhaponticum serratuloides FOR
Acroptilon repens
Acroptilon australe
ORIENTAL CLADE
Rhaponticum pulchrum RHA
Rhaponticum insigne
Rhaponticum helenifolium
Rhaponticum cynaroides STE
Rhaponticum acaule CES
Leuzea conifera LEU
Leuzea fastigueri LEU
Leuzea berardioides LEU
Rhaponticum exaltatum FOR
Rhaponticum canariense
Rhaponticum cassianum ELE
Rhaponticum longifolium FOR
Rhaponticum scariosum RHA
Rhaponticum scariosum (Slovenia)
OCCIDENTAL CLADE
RHAPONTICUM GROUP
Dark achene
Bright achene
North Africa (include Canary Island)
East Africa
Europe
Near East and Caucasus
Central Asia
East Asia
Australia
unresolved